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Full Length Research Paper

Statistical screening of factors affecting glucoamylase production by a thermotolerant *Rhizopus microsporus* TISTR 3518 using Plackett-Burman design

Jantima Arnthong[1], Boonpa Wanitchaploy[1], Kenji Sakai[2], Jean-Jacques Sanglier[3] and Vichien Kitpreechavanich[1,4]*

[1]Department of Microbiology, Faculty of Science, Kasetsart University, Thailand.
[2]Department of Plant Resources, Graduate School of Bioresource and Bioenvironmental Sciences, Kyushu University, Japan.
[3]Natural Products Unit, Novartis Institutes for BioMedical Research Basel, Novartis Pharma AG, Basel, Switzerland.
[4]Center for Advanced Studies in Tropical Natural Resources,KU Institute for Advanced Studies, Kasetsart University,Thailand.

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Glucoamylase is a key enzyme used in food processing as well as in commercial production of glucose from starch. The use of thermotolerant strain of *Rhizopus microsporus* TISTR 3518 offers the advantages of cooling-costs reduction during fermentation and high thermostable enzyme production. The effect of various carbon and nitrogen sources on glucoamylase production was evaluated. It was found that α-amylase treated liquefied cassava starch and CH_3COONH_4 gave the highest enzyme activity. The influence of various medium components and culture parameters were investigated using Plackett-Burman. It was shown that CH_3COONH_4, $FeSO_4.7H_2O$, $ZnSO_4.7H_2O$, $CaCl_2$, temperature and pH are significant factors affecting the glucoamylase production. The medium with the initial pH of 6.5 which consisted of α-amylase treated liquefied cassava starch, 10 gl^{-1}; CH_3COONH_4, 5 gl^{-1}; K_2HPO_4, 0.5 gl^{-1}; KCl, 1.5 gl^{-1}; $MgSO_4.7H_2O$, 0.5 gl^{-1}; $FeSO_4.7H_2O$, 0.06 gl^{-1}; $ZnSO_4.7H_2O$, 0.035 gl^{-1}; $CaCl_2$, 0.05 gl^{-1} and $C_6H_8O_7.H_2O$, 5.6 gl^{-1} yielded the highest enzyme production (948 U ml^{-1}) after cultivation at 40°C for 48 h.

Key words: *Rhizopus microsporus*, glucoamylase, Plackett-Burman design.

INTRODUCTION

Glucoamylase (EC 3.2.1.3.1; 4-D-glucan glucohydrolase) is a hydrolyzing enzyme acting on both α-1,4 and α-1,6 glucosidic links of starch. It can degrade both amylose and amylopectin and produce glucose (Elegado and Fujio, 1993). Nowadays, glucoamylase is one of the most important enzymes in food industries (Cook, 1982; Beuchat, 1987; Soccol et al., 1992), as it is used for the production of glucose and fructose syrup from liquefied starch (Nguyen et al., 2002). Glucoamylase are produced by various microorganisms, including bacteria, fungi and yeasts. Microbial strains of genera *Aspergillus* and *Rhizopus* have been mainly used for commercial production of glucoamylases (Pandey, 1995).

We recently characterized thermotolerant *Rhizopus* strains isolated from Thai traditional inoculum for alcoholic fermentation (Kitpreechavanich et al., 2008). The thermotolerant strain of *Rhizopus microsporus* TISTR 3518 produced more glucoamylase than the non-thermotolerant TISTR 3523 at 40°C. Additionally, the thermal stability of crude enzymes from *R. microsporus*

*Corresponding author. E-mail: fsciwck@ku.ac.th.

TISTR 3518 was also higher than that of the enzyme from TISTR 3523. The ability to grow and produce glucoamylase at high temperatures would be useful for cost-reduction in terms of cooling system.

For screening purpose, various medium components and culturing parameters were evaluated based on Plackett-Burman design which is a well established technique for selecting the critical culture variables (Gangadharan et al., 2007). This type of design is very economical as up to n factors can be evaluated in only n+1 combination. The aim of this study was to screen the factors affecting glucoamylase production by *R. microsporus* TISTR 3518 using Plackett-Burman design.

MATERIALS AND METHODS

Microorganism and cultivation

The *R. microsporus* TISTR 3518 strain isolated from Thai traditional inoculum was used in this study (Kitpreechavanich et al., 2008). The culture was maintained on a potato dextrose agar (PDA) slant at room temperature. Spore suspension was prepared from 7 days old culture that was grown on PDA slants, by aseptically adding sterile distilled water containing 0.1% tween-80 and then lightly brushing the mycelium with a sterile wire loop. The suspension was diluted with sterile distilled water to give a final spore count of 1 x 10^7 spores ml^{-1} determined by haemacytometer counts and was used as inoculums. Fifty milliliters of the medium in a 250 ml Erlenmeyer flask was inoculated with the inoculum and then shaked on a rotary shaker under 150 rpm at 35°C for 30 and 48 h. At the end of the cultivation, the mycelium was separated from the culture broth by filtering through a filter paper (Whatman no.1) to obtain the crude enzyme for glucoamylase activity assay.

Effect of carbon and inorganic nitrogen sources

Effects of carbon and nitrogen sources on glucoamylase production were studied in the basal medium with initial pH of 6.0 on a rotary shaker under 150 rpm at 35°C. The basal medium consisted of K_2HPO_4, 1 gl^{-1}; KCl, 1 gl^{-1}; $MgSO_4.7H_2O$, 0.5 gl^{-1}; $FeSO_4.7H_2O$, 0.01 gl^{-1}; $ZnSO_4.7H_2O$, 0.003 gl^{-1}; $CaCl_2$, 0.21 gl^{-1} and $C_6H_8O_7.H_2O$, 3.3 gl^{-1} (Morita et al.,1998). Five carbon sources (1.0% w/v): α-amylase treated liquefied cassava starch, glucose, maltose, soluble starch and cassava starch, and inorganic nitrogen source (0.7265 gl^{-1} N): CH_3COONH_4, $(NH_4)_2SO_4$, $NH_4H_2PO_4$ and $(NH_4)_2HPO_4$ were used. Glucoamylase activity was determined after 30 and 48 h of cultivation. A slurry of 20% (w/v) cassava starch in distilled water was liquefied by 120 KNU α-amylase gl^{-1} (Termamyl 120L, NOVO NORDISK, Denmark) at 90 - 95°C for 2 h with intermittently agitation. KNU is kilo Novo units of alpha-amylase and is equivalent to amount of enzyme which breaks down 5.26 g starch per hour. After liquefaction, a clear liquefied solution was obtained by filtering the slurry through a cheesecloth.

Plackett-Burman design

Initial screening of the most significant fermentation parameters that affected glucoamylase production by *R. microsporus* TISTR 3518 was performed by Plackett-Burman design (Plackett and Burman, 1946). The best carbon source, α-amylase treated liquefied cassava starch (X_1); best nitrogen source, CH_3COONH_4 (X_2); each components of the basal medium, K_2HPO_4 (X_3); KCl (X_4); $MgSO_4.7H_2O$ (X_5); $FeSO_4.7H_2O$ (X_6); $ZnSO_4.7H_2O$ (X_7); $CaCl_2$ (X_8); $C_6H_8O_7.H_2O$ (X_9) and physical factors, temperature (X_{10}) and pH (X_{11}) were considered as independent variables in this study. Each variable is represented at two levels: -1 for low level and +1 for high level. Table 2 shows the factors under investigation as well as levels of each factors used in the experimental design, whereas Table 3 represents the design matrix. The media were adjusted according to the experimental design. The spore suspension was inoculated into the medium and then incubated on a rotary shaker under 150 rpm at temperature according to the experimental design for 48 h. All the experiments were performed in triplicate and the average of glucoamylase production was used as the experimental response (dependent variable). The experimental design and obtained data were analyzed statistically using SPSS window 11 (version 11.5, 2002; USA).

Glucoamylase assay

The reaction mixture containing 0.5 ml of 2% (w/v) soluble starch in a acetate buffer 0.1 M, pH 4.5 and 0.5 ml of diluted crude enzyme, was incubated at 40°C for 20 min. The reaction was then stopped in a boiling water bath for 10 min. The amount of liberating glucose was measured by the glucose oxidase method (Kingsley and Getchell, 1969). One unit of glucoamylase activity was defined as the amount of enzyme required for releasing 1 µg of glucose per minute.

RESULTS AND DISCUSSION

Effect of carbon and inorganic nitrogen source

In the investigation of the effects of various carbon sources on glucoamylase production (Table 1), α-amylase treated liquefied cassava starch was the best carbon source with regard to the highest glucoamylase production. Gomes et al. (2005) reported that cassava starch was shown to be a better substrate than corn starch for glucoamylase production by *Aspergillus flavus*. The α-amylase serves to reduce the viscosity of gelatinized starch solutions and also to produce a lower molecular size substrate. These smaller substrate molecules are efficient inducers of glucoamylase which hydrolyzes the dextrins to glucose (Hockenhull, 1967). The production of glucoamylase by this strain using cassava starch which is abundant and cheap raw material will reduce cost of the enzyme production. Nitrogen source is the important nutrient for the growth of fungi and production of glucoamylase (Mamo and Gessesse, 1999). In our study, it was found that ammonium acetate was the best nitrogen source for glucoamylase production by *R. microsporus* TISTR3518 (Table 1). Ammonium acetate is provided not

Table 1. Effects of various carbon and nitrogen sources on glucoamylase production by *R. microsporus* TISTR 3518.

Carbon and nitrogen source	Glucoamylase activity (U ml^{-1})	
	30 h	48 h
Carbon sources		
Glucose	14	45
Maltose	141	380
Soluble starch	43	307
Cassava starch	9	349
Liquefied cassava starch	202	539
Inorganic nitrogen source		
CH_3COONH_4	223	540
$(NH_4)_2SO_4$	186	300
$NH_4H_2PO_4$	194	350
$(NH_4)_2HPO_4$	142	338

Table 2. Experimental variables at different levels used for glucoamylase production by *R. microsporus* TISTR 3518 using Plackett-Burman design.

Variable code	Variable	Level	
		Low (-1)	High (+1)
X_1	Liquefied cassava starch (gl^{-1})	10	30
X_2	CH_3COONH_4 (gl^{-1})	5	10
X_3	K_2HPO_4 (gl^{-1})	0.5	1.5
X_4	KCl ($g\ l^{-1}$)	0.5	1.5
X_5	$MgSO_4.7H_2O$ (gl^{-1})	0.5	1.5
X_6	$FeSO_4.7H_2O$ (gl^{-1})	0.02	0.06
X_7	$ZnSO_4.7H_2O$ (gl^{-1})	0.005	0.035
X_8	$CaCl_2$ (gl^{-1})	0.05	0.15
X_9	$C_6H_8O_7.H_2O$ (gl^{-1})	1	5.6
X_{10}	Temperature (^oC)	30	40
X_{11}	pH	5.5	6.5

Table 3. Plackett-Burman experimental design matrix for glucoamylase production by *R. microsporus* TISTR 3518.

Run number	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	Y (U ml^{-1})
1	1	1	-1	1	1	1	-1	-1	-1	1	-1	20
2	1	-1	1	1	1	-1	-1	-1	1	-1	1	98
3	-1	1	1	1	-1	-1	-1	1	-1	1	1	208
4	1	1	1	-1	-1	-1	1	-1	1	1	-1	28
5	1	1	-1	-1	-1	1	-1	1	1	-1	1	400
6	1	-1	-1	-1	1	-1	1	1	-1	1	1	792
7	-1	-1	-1	1	-1	1	1	-1	1	1	1	948
8	-1	-1	1	-1	1	1	-1	1	1	1	-1	717
9	-1	1	-1	1	1	-1	1	1	1	-1	-1	0
10	1	-1	1	1	-1	1	1	1	-1	-1	-1	627
11	-1	1	1	-1	1	1	1	-1	-1	-1	1	218
12	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	134

Table 4. Statistical analysis of Plackett-Burman design for glucoamylase production by *R. microsporus* TISTR 3518.

Variable code	Variable	Coefficient estimate	Mean square	F-value	P-value
X_1	Liquefied cassava starch (gl^{-1})	-21.67	5633.33	0.914	0.440
X_2	CH_3COONH_4 (gl^{-1})	-203.50	496947	80.60	0.012[a]
X_3	K_2HPO_4 (gl^{-1})	-33.17	13200.33	2.14	0.281
X_4	KCl (gl^{-1})	-32.33	12545.33	2.04	0.290
X_5	$MgSO_4.7H_2O$ (gl^{-1})	-41.67	20833.33	3.38	0.207
X_6	$FeSO_4.7H_2O$ (gl^{-1})	139.17	232408.33	37.70	0.026[a]
X_7	$ZnSO_4.7H_2O$ (gl^{-1})	86.33	89441.33	14.51	0.063[a]
X_8	$CaCl_2$ (gl^{-1})	108.17	140400.33	22.78	0.041[a]
X_9	$C_6H_8O_7.H_2O$ (gl^{-1})	16.00	3072	0.50	0.553
X_{10}	Temperature (ºC)	103.00	127308	20.65	0.045[a]
X_{11}	pH	94.83	107920.33	17.50	0.053[a]

[a] Significant at $P < 0.1$.

only as nitrogen source but also as carbon source. In addition, final pH of the medium using acetate as nitrogen source were 7.8 and 8.4 after 30 and 48 h of cultivation, respectively, which were suitable for the enzyme production. In case of the other nitrogen sources, the final pH decreased to 4.5 - 5.3 and diminished the enzyme production. Our result was coherent with the work of Morita et al. (1998), which showed that ammonium acetate was the best nitrogen source in *Rhizopus* sp. MKU40 for glucoamylase production. Nevertheless, it was different from the previous reports, which found that $(NH_4)_2HPO_4$ was the best for the enzyme production by *Aspergillus fumigatus (*Cherry et al., 2004) and *Candida famata* (Mohamed et al., 2007).

Screening of variables using Plackett-Burman

The corresponding response, glucoamylase activity (*Y*), from the media which adjusted according to the experimental design matrix of variables, is shown in Table 3. The experiment (run no. 7) in which the medium was adjusted to pH 6.5 and consisted of α-amylase treated liquefied cassava starch, 10 gl^{-1}; CH_3COONH_4, 5 gl^{-1}; K_2HPO_4, 0.5 gl^{-1}; KCl, 1.5 gl^{-1}; $MgSO_4.7H_2O$, 0.5 gl^{-1}; $FeSO_4.7H_2O$, 0.06 gl^{-1}; $ZnSO_4.7H_2O$, 0.035 gl^{-1}; $CaCl_2$, 0.05 gl^{-1} and $C_6H_8O_7.H_2O$, 5.6 gl^{-1} yielded the highest enzyme production (948 U ml^{-1}), after cultivation at 40°C for 48 h. The resulting effect of the responses, the associated coefficient estimate, mean square, F-value and significant levels (P-value) were investigated (Table 4). It was found that the P-value of six variables: X_2, CH_3COONH_4; X_6, $FeSO_4.7H_2O$; X_7, $ZnSO_4.7H_2O$; X_8, $CaCl_2$; X_{10}, temperature and X_{11}, pH was less than 0.10. Thus, this indicated that they were significant on the glucoamylase production. The addition of iron, magnesium and zinc ions in the liquid medium was essential to growth and glucoamylase production of *Rhizopus* sp. KMU40, while calcium ions also stimulated its growth and glucoamylase production (Morita et al., 1999). The effect of pH is one of the most important factors for growth of microorganisms and glucoamylase production. Cherry et al. (2004) found that the optimum initial pH was 7.0 for glucoamylase production by *A. fumigatus.* The model had a coefficient of determination (R^2) of 0.996, which can explain 99.60% variability of the data. Plackett-Burman design is a powerful technique for screening important variables and has successfully been used by many workers (Xu et al., 2002; Kaur and Satyanarayana, 2005; Kumar and Satyanarayana, 2006).

CONCLUSION

The present study involved the effect of carbon and nitrogen sources on glucoamylase production by *R. microsporus* TISTR 3518 and the use of statistical design of experiment to optimize parameters for the glucoamylase production. The α-amylase treated liquefied cassava starch and CH_3COONH_4 gave the highest glucoamylase activity. Six variables: CH_3COONH_4, $FeSO_4.7H_2O$, $ZnSO_4.7H_2O$, $CaCl_2$, temperature and pH were also identified by the Plackett-Burman design as significant for glucoamylase production.

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